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Studies on the Relationship between Membrane Bound Calcium and Membrane Phosphorylation

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Summary

Calcium binding to isolated human erythrocyte membranes has been investigated using radioactive $^{45}Ca^{2+}$. cAMP stimulated calcium binding to human erythrocyte membranes in the presence of 1 mM ATP and 2 mM Mg. Stimulation of membrane phosphorylation and calcium binding to human erythrocyte membranes by 3.3 μM cAMP was about 20%. The cAMP concentration required to obtain half-maximal stimulation of calcium binding to erythrocyte membranes was found to be approximately 0.16 μM,while the concentration causing half-maximal stimulation of membrane phosphorylation in human erythrocyte membranes was 0.1 μM. Maximal cAMP stimulation of membrane phosphorylation as well as calcium binding to membranes was obtained at a concentration of 3.3 μM. There was a linear relationship between the increase in membrane phosphorylation and the increase in membrane bound calcium. Thus, it appears that cAMP-stimulated calcium binding to membranes is associated with increased phosphorylation of membrane proteins.

Introduction

The significant role of calcium in the maintenance of membrane structure and a number of membrane functions, has often been noted[3, 7, 17]. In the case of human erythrocytes, ATP* depletion has been shown to result in cellular calcium accumulation[2]. In addition, it has been observed that cell deformability is affected by changes in cellular calcium content[18]. It has been suggested that a calcium-activated ATPase[6] and/or protein kinase[5] may play a role in these phenomena. However, there has been no direct demonstration regarding the regulation of membrane bound calcium by ATPase and/or protein kinase.

Evidence for the regulation of human erythrocyte cell deformability and shape control through membrane phosphorylation and membrane bound calcium has been accumulating[9, 12, 13]. Past observations suggest that cAMP, when applied extracellularly, may have an influence on the deformability of human erythrocytes[10, 11] most likely through

* Abbreviations used in this paper : ATP, adenosine 5′-triphosphate ; cAMP, adenosine-3′, 5′-cyclic monophosphate ; cGMP, guanosine-3′, 5′-cyclic monophosphate ; cIMP, inosine-3′, 5′-cyclic monophosphate.

stimulation of membrane protein kinase[14]. By atomic absorption spectrometry, we have shown that extracellular cAMP increases the amount of membrane bound calcium in intact human erythrocytes loaded with calcium[16]. Under the same conditions, extracellular cAMP interaction with intact human erythrocytes resulted in increased membrane phosphorylation[16]. These observations suggest certain interrelationships among the effects of cAMP, membrane phosphorylation and calcium on human erythrocyte functions. However, results obtained in our study alone were insufficient to provide a model for the relationship between membrane phosphorylation and calcium binding to membranes since intact human erythrocytes loaded with calcium were used in the study.

Therefore, we have studied the effect cAMP on calcium binding to isolated human erythrocyte membranes using $^{45}Ca^{2+}$ and investigated the stoichiometric relationship between membrane bound calcium and the state of membrane phosphorylation in the presence of cAMP.

Materials and Methods

Materials.

cAMP, ATP, bovine serum albumin and protamine sulfate were obtained from Schwarz-Mann Co. and Sigma Chemical Co. $^{45}CaCl_2$ (14～23 Ci/g) and [γ-^{32}P]ATP (20～25 Ci/mmol) were obtained from International Chemical and Nuclear Co.

Double glass distilled water prepared from deionized water was used for the preparation of all reagents. Plastic containers were used for the storage of calcium containing solutions because of the considerable adsorption of calcium to glass.

Preparation of erythrocytes and erythrocyte membranes.

Blood from young healthy male donors (19～31 years old) was collected directly into heparinized vacutainer tubes. The blood was centrifuged at 4°C and washed five times with 10 mM Tris buffer containing 150 mM NaCl (pH 7.8). Each erythrocyte preparation was monitored with a cell count of erythrocytes, reticulocytes, and leukocytes including a leukocyte differential count.

The residual leukocyte count in the erythrocyte preparations was $0.01 \pm 0.002\%$, with $0.066 \pm 0.021\%$ reticulocytes. Membranes were prepared from washed erythrocytes essentially by a modification[4] of the method of Dodge *et al.*[1].

Membrane phosphorylation.

The degree of membrane phosphorylation was determined by measuring the amount of ^{32}P incorporated into erythrocyte membrane proteins. The reaction medium contained 40 mM histidine-imidazole buffer (pH 7.2), 120 mM KCl, 2 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (60～70 cpm/pmol), 3.3 μM cAMP (when included) and 420～480 μg erythrocyte membranes in a total volume of 0.3 ml. Incubations were performed at 30°C for 15 minutes. The reaction was terminated by addition of reaction mixture (100 μl aliquots) to 2.5 ml of ice cold 11% trichloroacetic acid solution together with 20 μl of 2.5% bovine serum albumin

solution. The amount of ^{32}P transferred to membranes was determined as described previously[15].

Calcium binding to human erythrocyte membranes.

After incubating membranes in the membrane phosphorylation medium described above at 30°C for 15 min, 1.5 μM $^{45}CaCl_2$ (500~600 cpm/pmol) was added to the phosphorylation medium together with 2.5 mM Tris-oxalate, and the incubation was continued for an additional 5 minutes at 0 °C. At the end of the incubation, 100 μl aliquots of the reaction mixture were transferred into polycarbonate tubes containing 0.9 ml of 10 mM Tris-HCl buffer (pH 7.2) and immediately centrifuged at 29,000×g for 10 minutes at about 7°C. After removal of the supernatant, membrane pellets were resuspended in 1.0 ml of 10 mM Tris-HCl buffer (pH 7.2) and centrifuged again under the same conditions. After centrifugation, pellets were transferred into scintillation vials and dried. The membrane bound $^{45}Ca^{2+}$ was determined in 10 ml of scintillation fluid (10 ml of toluene containing 10% (v/v) BBS-3 solubilizer (Beckman) and 0.4% (w/v) Omnifluor (New England Nuclear)) using an Aloka LSC-900 liquid scintillation spectrometer. Control assays were carried out using boiled membranes.

Chemical analysis.

Protein concentration was determined by the method of Lowry *et al.*[8].

Statistical methods.

All results are expressed as the means ± SE (standard error) unless otherwise noted. Statistical significance was determined by the student's t test based on paired data. Statistical significance was assigned if $p<0.05$.

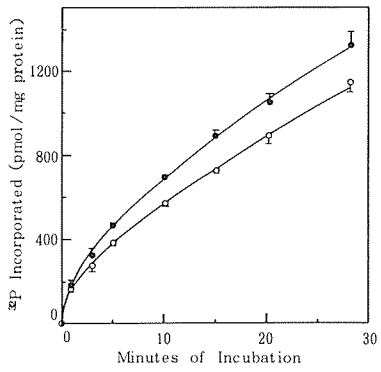

Fig. 1 Time course of phosphorylation of human erythrocyte membranes in the absence (○) and presence (●) of 3.3 μM cAMP at 30°C. Each point represents the mean ± SE from four experiments.

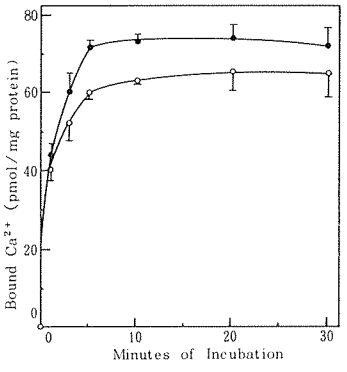

Fig. 2 Time course of $^{45}Ca^{2+}$ binding to human erythrocyte membranes at 0°C. The $^{45}Ca^{2+}$ binding assay was performed after incubating membranes in the membrane phosphorylation medium at 30°C for 15 min in the absence (○) and the presence (●) of 3.3 μM cAMP. Each point represents the mean ± SE from four experiments.

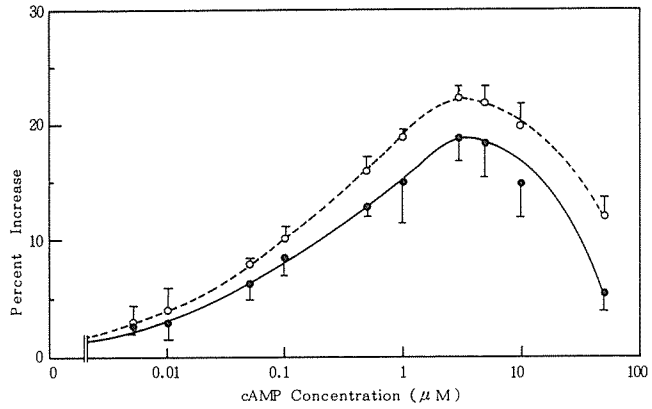

Fig. 3 Effect of cAMP concentration on human erythrocyte membrane phosphorylation (○) and $^{45}Ca^{2+}$ binding (●) to membranes. The assays were performed as described under "Methods". Values are the means ± SE from five experiments.

Results

cAMP stimulation of membrane phosphorylation and calcium binding to human erythrocyte membranes.

cAMP (3.3 μM) stimulated membrane phosphorylation as well as $^{45}Ca^{2+}$ binding to erythrocyte membranes (Figs. 1 and 2). Maximum stimulation (22%) of membrane phosphorylation by 3.3 μM cAMP was obtained at 30°C, 15 minutes after addition of cAMP. Significant stimulation (9.4%, $p<0.05$) of membrane phosphorylation was observed 1 minute after addition of cAMP, the earliest time measured. The binding of $^{45}Ca^{2+}$ to human erythrocyte membranes was a rapid process. The time required to reach half-maximal binding was 40 seconds to 1 minute at 0°C using 1.5 μM $^{45}Ca^{2+}$. Membranes phosphorylated in the presence of cAMP showed higher calcium binding capacity than those phosphorylated in the absence of cAMP (Fig. 2).

Concentration dependence of cAMP stimulation of membrane phosphorylation and calcium binding to membranes in human erythrocytes.

Figure 3 shows the dose-response curves for the effect of cAMP on membrane phosphorylation and $^{45}Ca^{2+}$ binding to membranes in human erythrocytes. At 3.3 μM, cAMP stimulation of $^{45}Ca^{2+}$ binding to human erythrocyte membranes was about 20%. The concentration of cAMP required to stimulate $^{45}Ca^{2+}$ binding to membranes half-maximally was graphically obtained to be approximately 0.16 μM. The cAMP concentration which gave half-maximal stimulation of membrane phosphorylation in human erythrocyte membranes by cAMP was 0.1 μM (Fig. 3). Maximal cAMP stimulation of membrane phosphorylation as well as $^{45}Ca^{2+}$ binding to membranes was obtained at a concentration of 3.3 μM.

Discussion

In this study using isolated membranes, we have focused on the relationship between membrane phosphorylation and membrane bound calcium stimulated by cAMP in order to contribute to an understanding of the consequences of cAMP stimulation of membrane phosphorylation.

We have found that cAMP stimulates calcium binding to isolated human erythrocyte membranes. Maximum increase in stimulation (22%) of membrane phosphorylation by 3.3 μM cAMP was obtained 15 minutes after addition of cAMP (Fig. 1). Membranes in which phosphorylation was stimulated by cAMP tended to bind more calcium than those phosphorylated in the absence of cAMP. This increase was observed over a range of incubation times, as seen in Fig. 2. Under the conditions employed here, there was no difference in the amount of calcium bound to membranes at 0°C or 30°C (Data not Shown).

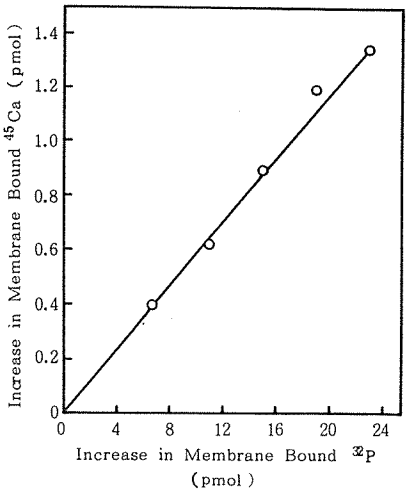

Fig. 4 Relationship of the increase in membrane phosphorylation and the increase in membrane bound $^{45}Ca^{2+}$ with varying protein concentrations. Membrane phosphorylation and $^{45}Ca^{2+}$ binding were determined with and without exposure to 3.3 μM cAMP as described in "Materials and Methods". The results are the means of duplicate experiments. The concentrations of membrane protein used for the assays were 50, 80, 110, 140, and 160 μg/ml.

The cAMP concentration required to obtain half-maximal stimulation of calcium binding to erythrocyte membranes was found to be 0.16 μM, a value comparable with the concentration of cAMP which stimulated membrane phosphorylation half-maximally (Fig. 3). The effect is specific for cAMP, although cGMP and cIMP could induce the effect to a much lesser degree (Data not shown). There was a positive correlation between the increase in membrane phosphorylation and the increase in membrane bound calcium under the influence of 3.3 μM cAMP, as shown in Figure 4. An increase in ^{32}P incorporation into erythrocyte membranes of approximately 15~16 pmol was associated with an increase of one pmol in membrane bound $^{45}Ca^{2+}$.

One might consider the possibility that phosphorylation could cause a decrease in membrane calcium content with consequent increased binding of $^{45}Ca^{2+}$ to unoccupied calcium binding sites. However, this is not likely since membranes isolated from human erythrocytes incubated with cAMP showed an increase in bound calcium as determined by atomic absorption[16]. In order to provide a distinct model for the relationship between membrane phosphorylation and membrane binding of calcium, it will be necessary to identify the specific membrane calcium binding sites whose calcium binding is stimulated by added cAMP.

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膜結合カルシウムと膜リン酸化の関連についての研究

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摘　　要

ヒト赤血球から単離した膜へのカルシウムの結合を$^{45}Ca^{2+}$をリガンドとして用い，調べた．1 mM ATP および2 mM マグネシウムの存在下，cAMP はカルシウムのヒト赤血球膜への結合を上昇させた．膜結合カルシウム量の最大増加は3.3 μM の cAMP で得られ，その増加量は約20%であった．同じく，3.3 μM cAMP の存在下で，膜リン酸化の上昇は最大（約20%）に達した．ヒト赤血球膜へのカルシウム結合の50%最大増加を引き起こす cAMP の濃度は約0.16 μM であった．一方，膜リン酸化の場合，50%最大上昇を引き起こす cAMP の濃度は約0.1 μM であった．また，ヒト赤血球膜リン酸化の上昇と膜結合カルシウムの増加との間には，直線的な比例関係が成立した．

したがって，cAMP によるヒト赤血球膜結合カルシウム量の増加は，膜リン酸化の上昇による可能性が考えられる．